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(54) Title: INDUCTION OF IMMUNE TOLERANCE BY IMMUNOTOXIN

(57) Abstract

A novel therapy for immunosuppression utilizes an immunotoxin to destroy the entire population of allograft reactive T cells during or after a tissue transplant procedure, followed by natural reconstitution of a tolerized T cell population. The therapy leads to permanent tolerance of the allograft thereby obviating the need for maintenance immunosuppressive drug therapy.

INDUCTION OF IMMUNE TOLERANCE BY IMMUNOTOXIN

10

FIELD OF THE INVENTION

          The therapy of the present invention relates to
the field of transplantation, and more particularly, to
15   a strategy for suppressing the immune response to an
allo- or xenograft, and then reconstituting the immune
system without further immunosuppression. The
reconstituted immune system is made tolerant to the
presence of foreign antigens, so that continuous
20   immunosuppressive drug therapy is not required.

BACKGROUND OF THE INVENTION

          In the field of organ transplantation,
25   implantation of donor tissue by allograft or xenograft
results in a cellular response leading to rejection.
The cells recognizing the histocompatibility antigens
of the graft belong to the class of T lymphocytes,
although other cell types are implicated
30   physiologically in the rejection process itself. It
has long been known that transplant patients require
immunosuppressive therapy to maintain healthy
functioning of the graft. The goal of this therapy is
to suppress T cell function just enough to block graft
35   rejection, but maintain immunity against infection.
          Several drugs have become available which
selectively suppress lymphocyte function without
appreciable toxicity and associated side effects in a
majority of patients. Typically, the degree of success

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expected, and the number and severity of side effects
is reflected in the closeness of match in
histocompatibility between host and donor tissue. In
some patients in which the tissue match is nearly
5   perfect, with only a mismatch at very minor
histocompatibility loci, a low dose of drugs is
indicated. These patients may have a slight depression
in peripheral white cell counts (down to 5.0 to 5.5/ml
compared to 6.0 to 6.5/ml), but will not display the
10   joint pain, swelling, and other symptoms of a more
significant mismatch or resulting from the
immunosuppression therapy itself.

Usually more than one drug is prescribed to obtain
the desired level of immunosuppression. The patient is
15   then monitored at least monthly to ascertain that a
suitable balance of drug concentrations is maintained,
and to check other parameters such as hematocrit, serum
creatine, white blood cell count, etc. Initially,
large, tissue-saturating doses of these drugs are
20   given, and the doses are then tapered off, until the
patient's condition stabilizes at an optimal dose.
Since rejection is mediated by infiltration of the
allograft with inflammatory cells, an anti-inflammatory
drug such as prednisone may also be given in addition
25   to the immunosuppressive agent or agents. For a review
of immunosuppressive drug monitoring combinational
strategies, see Rugstad, "Immunosuppressive Drugs", in
Immunopharmacology in Autoimmune Diseases and
Transplantation, eds. Rugstad, et al., Chapter 18, p.
30   233 et seq., Plenum Press, N.Y.: 1992.

The most commonly used immunosuppressive drugs are
Cyclosporin A (CyA) and thiopurines such as
azathioprine (AZA). Lesser immunosuppressive drugs
are: mercaptopurine, methotrexate, alkylating agents,
35   and glucocorticoids. CyA, marketed under the tradename
Sandimmune®, targets T-cells, in particular the helper
T-cell population which are reversibly inhibited. It
is not cytotoxic at therapeutic levels, does not

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disrupt DNA synthesis, and does not give rise to
lymphopenia. A major apparent impact is inhibition of
lymphokine production by preventing the cytosolic
components of transcription binding factors NF-AT and
5   NF-kappaB from translocating to the nucleus, thereby
inhibiting IL-2 gene transcription. It should be noted
that CyA does not act upon mature cytotoxic T
lymphocytes which are already differentiated, so that
inhibition of lymphokine production may lead to
10   inflammation.

Drugs, like CyA, with pronounced impact on normal
cell function can be expected to produce adverse
events. Among these are renal dysfunction such as
reduced renal plasma flow and glomerular filtration
15   rate. At higher doses, elevation in serum creatine and
histologic lesions in the kidney are noted. CyA
nephropathy has been described involving
arteriolopathy, acellular interstitial fibrosis, and
tubular atrophy.

20   The thiopurines, analogues of hypoxanthine, are
mildly toxic resulting in bone marrow aplasia and
hepatitis. Azathioprine, marketed as Imuran®, is
active as a metabolite, thioinosinic acid. While the
drug may cause some suppression of macrophage mediated
25   delayed hypersensitivity, its action is primarily
directed to T-cell dependent immune responses,
especially at low doses. This preferential effect on T
cells, with only slight inhibition of B cell responses
(possibly attributable in part to helper T cell
30   inhibition) accounts for its clinical popularity. The
corticosteroids can cause aseptic bone necrosis,
cataracts, and diabetes.

A very interesting group of immunosuppressants are
anti-T-cell monoclonal antibodies, which act either by
35   cell destruction or inactivation (receptor blockade),
or by immune modulation in which there is a
redistribution and disappearance of the target molecule
from the cell surface. One such antibody studied in

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the murine model is anti-L3T4, similar to the
corresponding antibody to the CD4 marker. Injection of
anti-L3T4 results in depletion of over 90 percent of
L3T4+ cells resulting in complete suppression of
humoral responses. Treatment with this antibody also
significantly extends allograft survival, but does not
result in tolerance (permanent non-recognition of the
allograft).

In primate models, it has been found that
monoclonal antibodies against the CD4+ and CD8+ T cell
subsets were immunosuppressive in monkeys receiving
renal or skin transplants. The mechanism appears to be
immune modulation, since there was no evidence of CD4+
cell depletion in animals treated only with OKT4
antibody. There was immunosuppression, in contrast to
studies in which only anti-CD8 antibodies were
injected. For an overview of the effects of monoclonal
antibodies directed to various lymphocyte markers
having a known or suspected role in allograft
rejection, see Chattenoud, et al., "Anti-T-cell
Monoclonal Antibodies as Immunosuppressive Agents", in
*Immunopharmacology in Autoimmune Diseases and
Transplantation*, supra, Chapter 14, p. 189 et seq.

Another interesting reagent, licensed for
immunosuppression therapy, is OKT3 antibody. OKT3 is a
pan-T-cell antibody capable of recognizing an antigen
on essentially all T cells. In conjunction with low
doses of other immunosuppressive agents, OKT3 reverses
acute allograft rejection in renal transplantation.
Prolonged administration of OKT3 results in complete
depletion of CD3+ cells so long as a detectable level
of OKT3 can be maintained in peripheral blood.
Eventually, there is a reappearance of CD3-CD4+ and
CD3-CD4+ antigen modulated cells; but no allograft
rejection occurs in the absence of CD3+ T cells.

The use of OKT3 and other anti-pan-T-cell
antibodies have the disadvantage of inducing
sensitization to the murine determinants. Once anti-

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OKT3 antibodies appear, there is a rapid elimination of
the agent from peripheral blood, thereby abrogating its
therapeutic value. In experiments with mice rendered
tolerant to rat species specific determinants, the mice
5 nevertheless developed high titers of anti-idiotypic
antibodies directed to the variable region. Thus, even
humanized forms of these antibodies may induce immune
responses if the variable complementarity directing
regions are not present in the human repertoire.

10   All of the drug or biologics based therapies
described above for immunosuppression have the
disadvantage of requiring lifelong continuous treatment
with a therapeutic regimen. Unless drug administration
is carefully monitored, imbalances can lead to a
15 spectrum of side effects. Too little suppression may
provoke a rejection episode; too much suppression can
cause leukopenia and other abnormalities of blood cell
numbers and ratios. As transplant procedures become
more routine, there is an increasing need for more
20 complete and systemic immunosuppression mechanism.

                    SUMMARY OF THE INVENTION

        In the present invention, permanent tolerance to
25 an allograft or xenograft is achieved by selectively
eliminating all T cell clones capable of mediating an
immune response to the foreign cells resulting in acute
rejection thereof. It is an object of the invention to
achieve permanent tolerance to the allograft without
30 the need for a transplant patient to take maintenance
doses of immunosuppressive drugs to control the
rejection process.
        A second object is to provide a therapy in which a
transient period of immunocompromise is short lived,
35 and does not affect the immunoglobin-secreting B
lymphocyte immunity already in place. A related aspect
involves maintaining the T-cell pipeline fully

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functional up to the last stages of maturation and
differentiation.

A further object is to provide a relatively non-
toxic reagent for inducing permanent tolerance to an
allograft or xenograft administrable in one or a few
bolus doses peritransplantationally.

The method of inducing immune tolerance to an
allograft in a mammal comprises administering a
therapeutically effective amount of an immunotoxin
consisting of restrictocin molecularly conjugated to an
monoclonal antibody having CD3-like binding
specificity. This method results in destruction of
essentially all T cells capable of mediating immune
rejections of transplanted tissue. The method is
further characterized in preferentially destroying all
such reactive T cells thereby depleting them, and then
allowing repopulation of the immune system by natural
regeneration of T cells in the absence of
immunosuppressive agents.

The reagent of the present invention is a
molecular conjugate which can induce permanent immune
tolerance towards allogeneic cells in a recipient
mammal comprising restrictocin covalently conjugated to
an antibody through a linkage cleavable under
physiological conditions within the target cells, and
an antibody contained in the conjugate having OKT3
characteristics such as affinity, marker specificity,
and toxin presentation and release properties.

The reagent is contained in an article of
manufacture comprising a sterile container of the type
used in the pharmaceutical art to contain an injectable
solution under pharmaceutical conditions of purity and
sterility, a solution of the reagent antibody-toxin
conjugate having the requisite antigen recognition
specificity corresponding to OKT3, and a label affixed
to the container bearing directions for administration
of the reagent in a dose sufficient to destroy
essentially all peripheral T cells outside of the bone

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marrow to thymus pathway, including all those T cells
having allograft rejection capability.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing the effect of
restrictocin on various mitogen stimulated
proliferation challenges of target T cells. Also shown
are data for mixed lymphocyte reactions.

Figure 2 is a rectilinear plot of the effect of
OKT3-restrictocin on mixed leukocyte proliferation at
various doses.

Figure 3 is a graph which plots the average
percentage of CD4, CD8 and CD45$^+$ positive cells in the
peripheral circulatory system against time expired
since PBL injection.

Figure 4 is a graph which plots the average
percentage of CD4, CD8 and CD45$^+$ positive cells in the
peripheral circulatory system and in the spleen against
time expired since PBL injection.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In a mammal, cellular immune responses are
mediated by T cell populations that recognize the
antigenic determinants present on transplanted
allografts. T cells are so named because they
represent a subpopulation of lymphocytes which
originate in the bone marrow and migrate through the
thymus gland where they mature into various subclasses
of cells. This maturation is a generally continuous
process, with continual regeneration of T cell
subpopulations from bone marrow derived stem cells. The
ontogeny of T cells is a very complex process, as yet
not well understood. However, it is believed that

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anti-self clones are eliminated in the thymus
epithelium by clonal deletion involving an abortive
interaction of various cell surface molecules including
CD3, CD4, CD8, one of the class of histocompatibility
5   antigens, and the T cell antigen receptor.

In acquired tolerance, both clonal deletion models
and clonal anergy have been demonstrated. Clonal
anergy is a state of down regulation of the clones,
which now become inactive in the presence of antigen.
10   The factors generally recognized to play a role in
tolerance induction are the immune competence of the
host, the structure and configuration of the antigen,
the immunogenicity of the antigen, the route of
administration, and the dose. In high zone tolerance
15   very large amounts of antigen induce a short-lived
paralysis of the immune response. Low zone tolerance
occurs upon administration of low doses of T cell
dependent antigens, is rapidly established, and
relatively long-lived. Clonal deletion models assume
20   that apoptosis of the emergent clone is achieved in the
epithelial microenvironment of the thymus, and that
anergy occurs in the peripheral lymph system after T
cell maturation has already occurred. Precisely what
mechanisms are involved in allograft tolerance induced
25   after massive depletion of the native immune system, as
in whole body irradiation is not completely known.

Reconstitution of the immune system with
autologous stem cells or donor allogeneic cells after
destruction of the native lymphocytes has been applied
30   in many situations involving blood cell neoplasms. (For
a review, see Fritz Bach, et al., *Transplantation
Immunology*, Wiley-Liss Inc.: 1995). Frequent
complications include graft versus host reactions, and
immunocompromised immune systems. This strategy has
35   been very effective in murine models to induce
tolerance of the allogeneic cells by eliminating all
the cells capable of recognizing the antigenic
determinants. In one interesting study, transgenic

mice expressing a cell membrane egg lysozyme were mated
to a second transgenic strain carrying a particular
anti-lysozyme IgM gene. B lymphocytes carrying the IgM
gene could not be detected, suggesting clonal deletion.

5   In the method of the present invention, tolerance
to an allograft (and potentially a xenograft) is
obtained by first destroying all T cells capable of
mediating an anti-allograft response, without killing
the regenerative source of stem cells in the bone

10   marrow. Newly regenerated stem cells will then migrate
to the thymus where T cell maturation takes place.
Because the allograft is now part of the body, the
immune system will perceive the allograft as self and
the reactive clones will either be depleted or

15   anergized.

          One of the Applicants hereof has recently
demonstrated the feasibility of this approach in a
rhesus monkey model in which renal allograft tolerance
was induced by prior treatment with a diphtheria-

20   derived toxin conjugated anti-T cell antibody (see
Knechtle, et al., _Transplantation_, 63: 1 (1997).
Several of the animals pretreated with the toxin-
conjugated antibody alone showed long term survival
without acute rejection, whereas all of the untreated

25   animals experienced acute rejection in 7-10 days. The
system described in this paper may be of limited use in
humans because the nearly universal presence of
neutralizing antibodies to the diphtheria-derived toxin
would quickly eliminate the therapeutic agent in

30   immunized populations. Also, the immunotoxin was not
uniformly effective, as some treated animals eventually
rejected their allografts, suggesting that some
reactive cells were spared in the pretreatment.   In
order to mitigate the toxicity of the diphtheria-

35   derived toxin, a mutant form having an altered binding
site was used. While still possessing potent protein
synthesis disrupting activity, it may be sufficiently
attenuated to permit some reactive cells to escape.

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Clearly, the completeness of T cell destruction is critical to the efficacy of this approach to achieving permanent tolerance to the allograft.

The immunotoxin of the present invention utilizes the toxin restrictocin molecularly conjugated to an antibody having the specificity of OKT3. OKT3 is a monoclonal antibody disclosed and claimed in U.S. Patent No. 4,361,549 hereby incorporated by reference, and developed by Ortho Pharmaceutical Corporation. It is preferred in the present invention for two reasons. First, it has the requisite specificity, recognizing essentially all differentiated T cells expected to be reactive to allogeneic cells. Secondly, it has already passed the scrutiny of the U.S. Food & Drug Administration. The antibody is licensed under the tradename Orthoclone OKT3 for use as an immunosuppressant in conventional therapy (see Physicians' Desk Reference, 1996, p. 1837 et seq.).

The power of monoclonal antibody technology is that every antibody molecule in the total clonal output has exactly the same primary structure, and therefore the same specificity for a single epitope. In the case of OKT3, specificity is directed to the CD3 epitope. This is an extremely interesting marker in the present application as it is believed that T cells only express this marker just at the point where the T cells are close to full differentiation and release from the thymus. This is important because it is desirable to clonally delete or suppress the allograft reactive cells, while at the same time, immunocompromising the patient to a minimal extent.

During the course of the present therapy, there will a short period of time in which the body will be somewhat vulnerable to challenge by pathogens having T cell dependent antigens. However, since essentially only peripheral T cells will be affected, there will be no disruption of the normal development of new T cells from bone marrow to the thymus. This means that

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restoration of complete immunocompetence will require
less time and involve less risk than if an early T cell
marker such as CD1 were selected. Since antibodies
having OKT3 specificity recognize only T cells and not
5   B cells, the ongoing and memory induced humoral
responses will not be affected by this therapy.

However, the antibody of choice in the present
invention is not limited to OKT3, but may be any
monoclonal antibody with substantially the same
10   specificity of OKT3. Such an antibody is herein
sometimes referred to as OKT3-like. By the term "same
specificity" Applicants mean an antibody having an
antigen binding specificity which recognizes the same
cell surface marker as OKT3, whether or not precisely
15   the same epitope, and which has sufficient binding
affinity, generally in the nanomolar (nM) range,
recognize and bind target T cells and discharge the
immunotoxin. Therefore, the term also means any
antibody having specificity for CD3 which presents the
20   toxin so that it is taken up into the cytoplasm.

The antibody may be of any class suitable to this
indication, preferably class IgG1. It may be a
recombinant form altered as to its species specific
components to become humanized (e.g. nonimmunogenic to
25   humans). The antibody may also be a stabilized,
truncated form, a single chain antibody, or
conventional enzymatically prepared fragments, such as
Fab, known in the art. These smaller forms may be
particularly preferred where non-specific tissue
30   interactions involving the Fc region are to be avoided,
and where the smaller forms may diffuse more readily
into the sites of T cells and T cell clones resident in
solid tissue. The preferred toxin in the present
invention is restrictocin, a fungal toxin secreted by
35   *Aspergillus restrictus*. Restrictocin is one of a class
of fungal toxins which have been evaluated as
conjugates for delivery to target cells in therapeutic
strategies. For an older, but informative overview of

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the field of plant and fungal proteins and glycoprotein
toxins affecting eukaryote protein synthesis, see
Jimenez, et al., *Ann. Rev. Microbiol.*, 39: 649 (1985).
Of the four most studied fungal toxins, alpha-sarcin,
5   mitogillin, aspf1, and restrictocin, restrictocin is
preferred for the following reasons: (i) extremely high
specificity and efficiency in cleaving 28s rRNA, (ii)
availability in commercial quantities of native,
recombinant, and mutant forms, (iii) apparent lower
10   toxicity to non-targeted cells of toxin dissociated
from its conjugate, and (4) ability to cross cell
membranes (see, for example, Yang, et al., Structure,
4: 837 (1996)).

One reason for restrictocin's superiority as an
15   immunotoxin is that the free molecule lacks a cell
binding domain, and requires a binding intermediary
such as an antibody, for entry into cells. Thus, an
unprotected bond between antibody and restrictocin can
be used to enhance cytoplasmic delivery without toxic
20   side effects. These side effects are obviated since
circulating free restrictocin released by
physiologically mediated sulfhydryl reduction will not
penetrate untargeted cells. Another reason for
restrictocin's superiority is that stable conjugation
25   requires only one linkage since restrictocin is a
single chain protein of 149 amino acids. Another
advantage to restrictocin is that it is a small
molecule compared to other toxins, and therefore less
immunogenic. A discussion of these advantages is set
30   forth in Rathore, et al., *Biochem. and Biophy. Res.
Comm.*, 222; 58 (1996).

Restrictocin may be obtained and purified by
essentially the original published method, as modified.
U.S. Patent No. 3,104,208, incorporated by reference,
35   disclosed a method for producing and purifying
restrictocin over 30 years ago. The organism (NRRL
2869) was grown in a rich undefined medium under
specified conditions of temperature and aeration. The

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supernatant fluid was applied to an Amberlite ICR-50
column, and the eluted restrictocin thus purified.
Further purification utilizing Millipore Pellicon
filtration was described by Yang, et al., *J. Gen.*
Microbiol., 138: 1421 (1992), hereby incorporated by
reference. In the Example of this application, a
further hollow fiber concentration step is followed by
HPLC. There are now available methods known in the art
of carrying out preparative HPLC or its equivalent for
production of commercial quantities of restrictocin
sufficiently pure to meet FDA QA/QC requirements.

Conjugation of restrictocin to OKT3-like antibody
requires an intermediate step since the native molecule
does not contain a free sulfhydryl group. Conde, et
al., *Eur. J. Biochem*, 178: 802 (1989), hereby
incorporated by reference, described the basic
procedure for derivatizing restrictocin with N-
succinimidyl 3-(2-pyridyldithio) proprionate (SPDP).
The OKT3-like antibodies are similarly derivatized with
the same agent. The purifications separating the
derivatives from the unreacted species are also
described in detail. It is also efficacious to
generate sulfhydryl groups via 2-iminothiolane.
Complete reaction specifications, reagents, and
procedures for utilizing various derivatizing and
conjugating strategies are set forth in G.T. Hermanson,
Bioconjugate Techniques, Academic Press: 1996), hereby
incorporated by reference. See especially Chapter 7,
"Cleavable Reagent Systems", pp. 292 et seq. for a
detailed description of conjugates which release the
conjugated toxin. While emphasizing the use of
cleavable systems in structure verification and moving
a molecule from one conjugate to another, it is
apparent from Conde, et al., supra, that these systems
can be used to produce immunotoxins directed against
human tumor cells.

In a preferred method of preparing and purifying
the immunotoxin conjugate, the reacted material is

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purified by double affinity chromatography, as
disclosed in detail in the Examples. Restrictocin-
specific antibodies are covalently bound to a column
matrix. This column is then used to bind the
5   restrictocin-conjugated OKT3-like antibodies. The
recovered, eluted conjugate is then purified again on
an affinity column which binds antibody. The final
eluant may then be finally purified and the product
confirmed by gel filtration. The double affinity
10   procedure results in a more highly purified conjugate
than conventional gel filtration alone. A relatively
small column bed can be used for even commercial scale
batches, since the bulk of material passes through the
column in a wash, and the amount of bound material is
15   small, particularly in the second step.
          One reason for refining the purification process
for preparing the immunotoxin as much as possible is
addressed in R.W. Baldwin, et al., eds., *Monoclonal
Antibodies and Immunoconjugates*, Parthenon Publishing
20   Group: 1990. In the tumor field it was found that the
presence of free antibody effectively competed for
sites on tumor cells, thus sparing some cells from the
lethal effect of the toxic conjugate. This led to much
shorter survival prolongation times than expected.
25   Improved purification procedures only marginally
improved this result, since it was found that free
antibody was generated readily from the natural
dissociation of the disulfide bonds holding the
conjugate together, under physiological conditions.
30   The article goes on to teach that the problem can be
overcome by utilizing another coupling agent N-
succinimdyl-oxycarbonyl-alpha-methyl-alpha-(2-
pyridyldithio)toluene (SMPT). The disulfide bond in
the conjugated is protected from thiol attack by the
35   presence of an adjacent methyl group and benzene ring.
The structures of these and other coupling agents
useful in biological applications are given in *Double-*

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Agents, Cross-linking Reagents, A Selection Guide,
published by Pierce Chemical Company, 1996.

The prior discussion here of the problems of
adapting immunotoxins to the field of oncology
emphasizes the contrast to the requirements of
tolerance induction in the field of transplantation.
Contrary to the above teaching, it is an advantage to
use an unprotected disulfide link between the toxin and
antibody, because the ease of dissociation under
particularly intracellular conditions helps to ensure
complete penetration of all target T cells with high
efficiency. As set forth above, the premature release
of free restrictocin in small amounts will not produce
prohibitively debilitating side effects, as with alpha-
sarcin, because it is potent in lower levels generally,
and the free restrictocin has no cellular binding
domain.

In the therapeutic method of the present
invention, the restrictocin-OKT-like purified conjugate
is administered, preferably by intravenous injection,
in a therapeutically effective amount. The blood
levels of free restrictocin, and the conjugate can be
monitored by conventional assays, and the blood level
of the conjugate maintained at a threshold level of .01
to 15 ng/ml, and generally in a range empirically
determined to result in complete tolerance to the
transplant. Thus, a therapeutically effective amount
is that level of the conjugate which saturates the
peripheral blood and tissues.

The administration of the total amount may be a
bolus given at one or several times, or a continuous IV
administration peritransplantation, beginning prior to
the actual transplant, either accompanied or not by
intrathymus infusion of the donor cells or an extract
thereof, and continuing for several days thereafter.
The amount given should at least be sufficient to
prevent emergence from the thymus of new differentiated
T cells during treatment. In general, the treatment

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regimen for use of OKT3 in immunosuppression may be a
guide. This regimen consists of a 5 mg bolus dose
administered on each of 10 to 14 days post rejection
episode.

5   Another advantage of the present method is that it
may be used with patients who have already undergone
transplant, and are repelling allograft rejection by
maintenance doses of conventional immunosuppressive
drugs. So long as there is a continuous source of
10   allogeneic cells, and the dose administered
peritransplantally, of restrictocin-OKT3 conjugate is
therapeutically effective as herein defined, there is
no distinction as to the timing of the allograft or
xenograft, whether it be substantially simultaneous, or
15   occurring at some remote time.

Further advantages of the present invention will
be apparent from the Examples which follow.

                           EXAMPLE 1
20

Production of Restrictocin.

Restrictocin is obtained from the growth media of
the fungus Aspergillus restrictus propagated in a
liquid medium fermenter. The medium can be minimal or
25   complex, with the important feature that the fungus
must be cultivated for a sufficient period of time that
it will undergo conidiation in solution.

There should be sufficient nutrient that it will
continue to metabolize after it has undergone most of
30   its growth and begun to exhaust some of the nutrient in
the medium. An important aspect of minimal growth on
acetate and ammonium as the carbon and nitrogen sources
is to control the pH at 7.6 where the conidiation seems
best and growth can continue. The production of
35   restrictocin can be monitored via HPLC or by
immunoblots, as described in Brandhorst, Yang and
Kenealy, "Heterologous Expression of the Cytotoxin
Restrictocin in Aspergillus Nidulans and Aspergillus

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Niger", *Protein Expression and Purification*, 5, 1994,
486-497.

EXAMPLE 2

Purification of Restrictocin.

The hyphal material is separated from the culture
filtrate by coarse filtration through Whatman filters
or cheesecloth, miracloth or the like. The hyphal
material is washed to remove residual restrictocin and
then the filtrates pooled. The following steps can be
done in any order.

Cation exchange chromatography.   The filtrate is
applied to the column (no adjustment in pH if between
pH 5.0 and 8.0). The salt content of the medium should
be below that of 0.75M NaCl or its equivalent in ionic
strength. The preferred resin, Amberlite IRC-50, is
described in U.S. patent nos. 3,104,204, 3,104,208, and
3,230,153. The resin is prepared first by soaking in
1N NaOH (with constant mixing) followed by a rinse in
distilled or deionized water followed by neutralization
with dilute phosphoric acid until the suspending
aqueous solution above the resin remains between pH 7.0
and 5.0. At this point the resin can be loaded into a
column and rinsed with 10 columns volumes (or more) of
distilled or deionized water.

Once the filtrate is applied to the column, the
column is eluted with distilled water until the
absorbance trace on a UV monitor decreases to baseline.
The column is then eluted with 0.5 M NaCl followed by a
gradient or step elution to a concentration of 5 M
NaCl. The UV-absorbing material (280nm) is collected
and peaks are pooled. The restrictocin containing
peaks (determined via HPLC or immunoblots) are
collected and subjected to Ultrafiltration.

Ultrafiltration.   The pooled peaks from the
column or the filtrate directly is filtered on an AGT
30,000 NMW hollow-fiber filter. The filtrate

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(permeate) is collected and the retentate is washed
several times with distilled water with the filtrate
being pooled together. This procedure removes proteins
of greater than 30,000 MW and the restrictocin should
pass through the filter matrix. The hollow fiber
cartridge is then cleaned with 0.5N NaOH and distilled
water and stored in 30% ethanol at 0-10 C. If the
filtration is done first the filtrate is then put onto
the column above. If the filtration is done after the
column chromatography then the filtrate is concentrated
and diafiltered as below.

Concentration and diafiltration. This step is
best done after the filtration above. It will be
required eventually after the chromatography since
there will be large amounts of salt in the solution.
The hollow fiber cartridge AGT 3000 NMW is taken out of
storage and the ethanol washed away with distilled
water. The pump and holding tank are also well rinsed
prior to use. The filter is used to concentrate the
material and the concentration of salt in the solution
is reduced by adding distilled water to the reservoir
holding the retentate. This effectively retains the
protein of interest and by determining the hold up
volume and dilution the amount of salt present can be
calculated and reduced in the final retained material.
The filtrate from this material is usually discarded.
When the amount of salt is considerably less than the
amount of restrictocin the concentration is brought to
its lowest volume and the material recovered from the
filter and associated piping rinsed with distilled
water. All the protein containing washes are pooled
freeze-dried.

Reverse Phase HPLC. The material treated with all
the steps above is then purified to homogeneity by C-4
reverse phase chromatography (1). The fractions from
the chromatography are quickly brought to dryness using
a cool rotary concentrator and then rehydrated using
distilled water. If this is not done quickly enough

there will be some degradation of the protein and some
will precipitate out of the solution. The
trifluoroacetic acid and acetonitrile are removed in
this drying step. The resuspended protein can have a
5   small amount of buffer added to it to neutralize the
acidic groups on the protein. This material can be
frozen and stored or freeze-dried and stored until used
further.

         Activity testing. When the protein is pure an
10 approximate extinction coefficient of A280=1.0 for a 1
mg/ml solution can be used to estimate the protein
concentration. The material purified in the above
manner is tested for its ability to generate the alpha-
fragment from ribosome preparations, as described in
15 Brandhorst and Kenealy, supra. This must be done to
assure the activity of the protein is present and that
the protein is active as a toxin.

                    EXAMPLE 3

20

Conjugation Methods.

         Restrictocin does not contain any free sulfhydryls
as purified. The four cysteines present in
restrictocin are coupled together in two disulfide
25 bonds. The conjugation methods thus far attempted have
focused on using a disulfide link between the antibody
and restrictocin to hold the molecules together. This
is advantageous since the disulfide bond is one that is
relatively easy to cleave inside cells. Thus the toxin
30 and antibody are connected in a bond that is designed
to come apart after the toxin is delivered to the
cells. The antibodies made usually do not have free
sulfhydryls present and both proteins have to be
modified to form the desired bond.
35   For purposes of this synthesis the restrictocin is
modified to have a free sulfhydryl and the antibody
modified so that a sulfhydryl is available that is
protected with a good leaving group. This will allow

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the exchange of a free sulfhydryl for the leaving group
and the bond between the antibody and restrictocin will
be formed.

5   The methods used are similar to these outlined in
Hermanson, G.T., *Bioconjugate Techniques*, Academic
Press: San Diego, CA, 1996. Restrictocin is modified
by adding SPDP which binds to free primary amines.
Reactions are also planned for sulfhydryl generation
via 2-iminothiolane. The molar ratio of restrictocin
10   to SPDP is one to one which means that some
restrictocin will not be modified and some will be
modified twice. After this first attachment of SPDP
the modified restrictocin is reduced with
dithiothreitol and excess reactants are removed via
15   filtration on a 3-5,000 NMW filter or via gel
filtration to desalt the restrictocin (for 2-
iminothiolane the reduction is not done since the
reaction provides a free sulfhydryl). The buffer at
this point is roughly neutral in pH and contains EDTA
20   to inhibit metal catalyzed oxidations of the free
sulfhydrls. The restrictocin preparation is washed on
the filter with this buffer and then removed from the
filter to estimate the concentration of restrictocin
(280nm).

25   The antibody is modified in a similar manner
except the molar ratio of SPDP in the initial reaction
is 3 SPDP to 1 antibody. Also the reduction does not
take place, instead the preparation is filtered using a
100,000 NMW filter and washing with the above EDTA
30   containing buffer. At this point that antibody is
removed from the filter and the concentration
estimated. A molar ratio of 3-6 restrictocins to one
antibody is mixed together and allowed to react. After
sufficient time has elapsed the mixture is filtered on
35   the 100,000 NMW filter and rinsed with the above
buffer. This mixture contains a variety of forms of
the proteins. There will be some unconjugated

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restrictocin and antibody remaining along with several
ratios of restrictocin to antibody conjugates.

EXAMPLE 4

<u>Conjugate Purification</u>.

       The conjugate is purified by size, and by affinity
chromatography. The filtration and washing on the
100,000 NMW filter, if done extensively, above can
remove all of the unreacted restrictocin. However the
best purification uses affinity chromatography for both
the antibody and restrictocin. Antibodies that bind
restrictocin are generated in rabbits or chickens,
affinity purified using restrictocin bound to column
and then the antibodies are eluted and conjugated to a
second column. The antibodies will bind restrictocin
and not the monoclonal antibody. So only the
conjugates with restrictocin attached and any free
restrictocin will bind to the column. The bound
material is eluted from the column, the eluting agent
neutralized either by filtration or drying and then the
material is again affinity purified on a column that
binds the antibody portion of the conjugate. Once the
bound material is washed and eluted off the second
affinity column the only compounds in solution should
have restrictocin and antibody coupled together.

       This material can be used as a test material or it
can be further refined using a size fractionation which
should separate the 1:1 2:1 etc. ratios of restrictocin
to antibody from each other. The conjugates will also
be analyzed by polyacrylamide gel electrophoresis in
both a reducing form and non-reducing form. Reduced
samples will show the subunits that go to make up the
antibody and the amount of restrictocin present. The
non-reducing samples will indicate the size of the
whole conjugate.

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EXAMPLE 5

The efficacy of the restrictocin-OKT3 was
evaluated in a series of invitro tests designed to
5   determine the completeness of blocking T cell responses
to a wide range of T cell stimuli. Inhibition of
proliferative response in mixed leukocyte tests were
also carried out to determine effective invitro doses
required for complete ablation. The tests were carried
10   out according to the following protocol:
Human peripheral blood leukocytes were isolated by
ficoll hypaque which separated the population of
reactive T cells (and other white blood cells such as B
lymphocytes and monocytes) from granulocytes and
15   residual red blood cells. In certain experiments, non-
tissue culture treated EIA plates were precoated with
soluble antigens or mitogens for 24 hours prior to
addition of responding cells (EIA plates, Costar #
3791). An identical plate was established in tissue
20   culture-treated plates by adding antigen at the time of
cell input (TC plates, Costar #3799). In other TC
plates, mitogens and polyclonal stimuli were added to
responder cells at optimal concentrations. After three
days, all culture wells were harvested of 100 ul of
25   media to test for production of cytokines, while the
remaining cells were split into two plates; one to be
assayed for viability by MTT dye technique, the other
was pulsed with 3H-thymidine for 18 hours.
Detection of cytokine release was achieved by
30   ELISA (Endogen and R & D matched pair kits), while MTT
metabolism was performed by solubilizing the
metabolized tetrazolium crystals in solubilization
buffer. In both cases color differences were
determined by microplate ELISA reader (Biotek).
35   Thymidine incorporation was determined by harvesting
cell contents, washing, a counting in a scintillation
counter (Matrix 96, Packard Instruments).

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The mixed lymphocyte reactions were run in two
batches to test the concentrations required to suppress
proliferation. The results are tabulated in Tables 1A
and 1B. The results of batch 1 are also plotted in
figure 2. It is apparent that the OKT3 antibody alone
exerts some stimulatory effect on mixed cells at high
concentrations. This effect is not completely abated
until concentrations of antibody are reduced from 1000
ng/ml to less than 30 ng/ml. In contrast,
concentrations of as little as 250 ng/ml of OKT3
conjugated to restrictocin are effective in preventing
essentially any T cell stimulation, and some
suppression is noted even at concentrations as low as 4
to 8 ng/ml.

In batch 2 (Table 1B), the effect of OKT3-
restrictocin was compared at two levels of purity. A
"crude" preparation was compared to one involving an
extra column purification step. No differences were
detected, indicating that the presence of small amounts
of free reagent do not interfere with the suppressive
effect under invitro conditions.

Figure 1 is a bar graph plotting a comparison of
antigen and mitogen stimulation under conditions of
treatment with OKT3-restrictocin, DAB-486, which is a
highly toxic positive control consisting of a molecular
construct of IL-2 and diphtheria toxin C chain, and an
untreated control containing media only. The results
clearly and dramatically show that OKT3-restrictocin
inhibition of mitogen stimulated proliferation is
essentially complete at the minimum inhibitory dose
established in the batch experiments described above.
The inhibitory effect was, in fact, more complete than
for DAB-486, and was evident for all antigen/mitogens
tested (from left to right, media only control,
concanavalin A, High IL-2 (100 units), Poke Weed
mitogen, Phabal myristate acetate, lipopolysaccharide,
phytohemagglutinin, and the mixed lymphocyte reaction.
This is expected to correlate with essentially complete

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suppression and elimination of allograft reactive clones in the T cell population in vitro.

EFFECT OF OKT3-RESTRICTOCIN ON MLR PROLIFERATION

Table 1A

[CONC]	OKT3 mAB		T3-RESTRICT. BATCH 1	
(ng/ml)	MEAN	STD	MEAN	STD
1000	67.1	4.4	0.0	0.0
500	66.2	2.7	0.1	0.0
250	56.1	4.8	1.1	0.9
125	47.6	6.1	11.3	1.1
63	38.7	0.8	15.9	3.0
31	32.2	4.7	8.2	3.4
16	27.0	1.3	14.7	4.4
8	27.2	0.9	6.9	3.0
4	27.8	3.2	14.5	4.2
2	27.3	13.7	19.1	4.1
PBS VEH	25.2	3.6	21.5	3.7
UNTREAT	25.9	5.7	24.1	0.1

RESP = AL G.   STIM = DEB MACK

Table 1B

[CONC] (ng/ml)	T3-RESTRICT. BATCH 2		[CONC] (%)	T3-RESTRICT. CRUDE	
80	8.6	1.0	20.0%	19.6	2.6
40	10.4	3.9	10.0%	27.5	0.5
20	17.6	3.8	5.0%	26.7	0.4
10	21.4	1.2	2.5%	21.2	0.2
5	19.1	2.2	1.25%	19.4	2.8
2.5	27.4	3.0	0.63%	21.6	0.6
1.25	19.2	3.0	0.31%	22.1	2.1
.63	22.3	5.0	0.16%	20.6	4.3
.31	26.3	5.2	0.08%	23.4	3.6
.16	33.8	1.2	0.04%	19.1	3.8
PBS VEH	32.8	5.1	0.02%	22.2	0.6
UNTREAT	27.6	8.7	UNTREAT	26.2	9.5

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EXAMPLE 6

The efficacy of OKT-3 restrictocin in decreasing
the number of human T-cells in vivo was examined.
Briefly, on Day 1, SCID mice were injected with $1X10^8$
human peripheral blood lymphocytes (PBL). On day 8, a
piece of human skin of full thickness was grafted onto
the SCID mice. On Day 9, the mice were injected with
$5\mu g$ OKT-3 restrictocin or with PBS as a negative
control. At 3, 4 and 7 weeks post-PBL injection, flow
cytometry was used to determine the extent of
reconstitution of human CD4, CD8 and CD45^{+} cells (T
cells). The data are presented in Figures 3 and 4.
    With respect to skin graft rejection, no
difference was seen between control and treatment
groups. This is probably because in this experimental
system rejection of the human skin can be masked by
extensive vascularization of the skin graft with mouse
endothelium.
    This experimental system allows the determination
of the effect of treatment on T cell numbers. Figure 3
is a graph which plots the average percentage of CD4,
CD8 and CD45^{+} positive cells in the peripheral
circulatory system against time expired since PBL
injection (Im Tx- OKT-3 restrictocin treated SCID mice,
NC- negative control PBS treated mice.) These data
demonstrate that OKT-3 restrictocin treatment
administered in only a single dose dramatically reduces
the number of T cells in PBL injected SCID mice. The
suppression of T-cell reconstitution is greatest at
three weeks and still evident at four weeks post PBL
injection.
    Figure 4 is a graph which plots the average
percentage of CD4, CD8 and CD45^{+} positive cells in the
peripheral circulatory system and in the spleen against
time expired since PBL injection (Im Tx- OKT-3
restrictocin treated SCID mice, NC- negative control
PBS treated mice.) The data for the first two time

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points is identical to the data presented in Figure 3.
These data demonstrate that after seven weeks the
number of T cells in the spleens of OKT-3 restrictocin
treated mice is greatly reduced as compared to control
5   mice.

In summary, the single dose OKT-3 restrictocin
treatment of PBL injected SCID mice produced a dramatic
decrease in T cell numbers (about 50%). These results
lead to the expectation that multiple dose treatments
10   with OKT-3 restrictocin will decrease T cell numbers to
a much greater extent. The desirablity of using OKT-3
restrictocin for the induction of immune tolerance is
further demonstrated by the fact that no clinical
toxicity was observed at the dose which produced the
15   dramatic decrease in T cell numbers. The results
indicate OCT-3 functions as an effector for delivery
and selective uptake of restrictocin into targeted
cells without appreciable uptake by unselected cells
leading to toxicity. The inclusion of selective uptake
20   is unknown and fortuitous.

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INDUCTION OF IMMUNE TOLERANCE BY IMMUNOTOXIN

CLAIMS

What is claimed is:

1.   A method of inducing immune tolerance to an allograft in a mammal comprising
        administering a therapeutically effective amount of an immunotoxin consisting essentially of restrictocin molecularly conjugated to an antibody having OKT3-like binding specificity.

2.   A method of inducing immune tolerance to histoincompatible transplanted tissue in a mammal comprising
        administering restrictocin conjugated to an antibody having substantially the binding specificity of OKT3 in an amount effective to destroy essentially all T cells capable of mediating immune rejection of said transplanted tissue.

3.   A method of inducing sustained tolerance of an allograft without continuous immunosuppression thereafter comprising
        administering to a mammal restrictocin conjugated antibody having the T cell antigen recognition specificity of OKT3 in a dose sufficient to deplete all antiallograft reactive T cells, and
        repopulating the immune system by natural regeneration of T cells in the absence of immunosuppressive agents.

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4.   A molecular conjugate useful for inducing
permanent immune tolerance towards allogenic cells in a
recipient animal comprising
        restrictocin covalently conjugated to an antibody
through a cleavable site, and
        an antibody contained in said conjugate having
OKT3 like characteristics.

5.   The method of claims 1, 2, and 3 wherein said
conjugated antibody is administered concurrently with a
transplant procedure.

6.   The method of claims 1, 2, and 3 wherein said
conjugated antibody is administered at any time after a
transplant procedure.

7.   An article of manufacture comprising
        a sterile container of the type used in the
pharmaceutical art to contain an injectable solution
        a solution contained in said container comprising
a restrictocin conjugated antibody having the T cell
antigen recognition specificity of OKT3, and
        a label affixed to said container bearing
directions for administration of said conjugated
antibody to a mammal in a dose sufficient to destroy
essentially all T cells having allograft rejection
capability.

8.   The method of claims 1 to 5 wherein said
restrictocin conjugated antibody is conjugated through
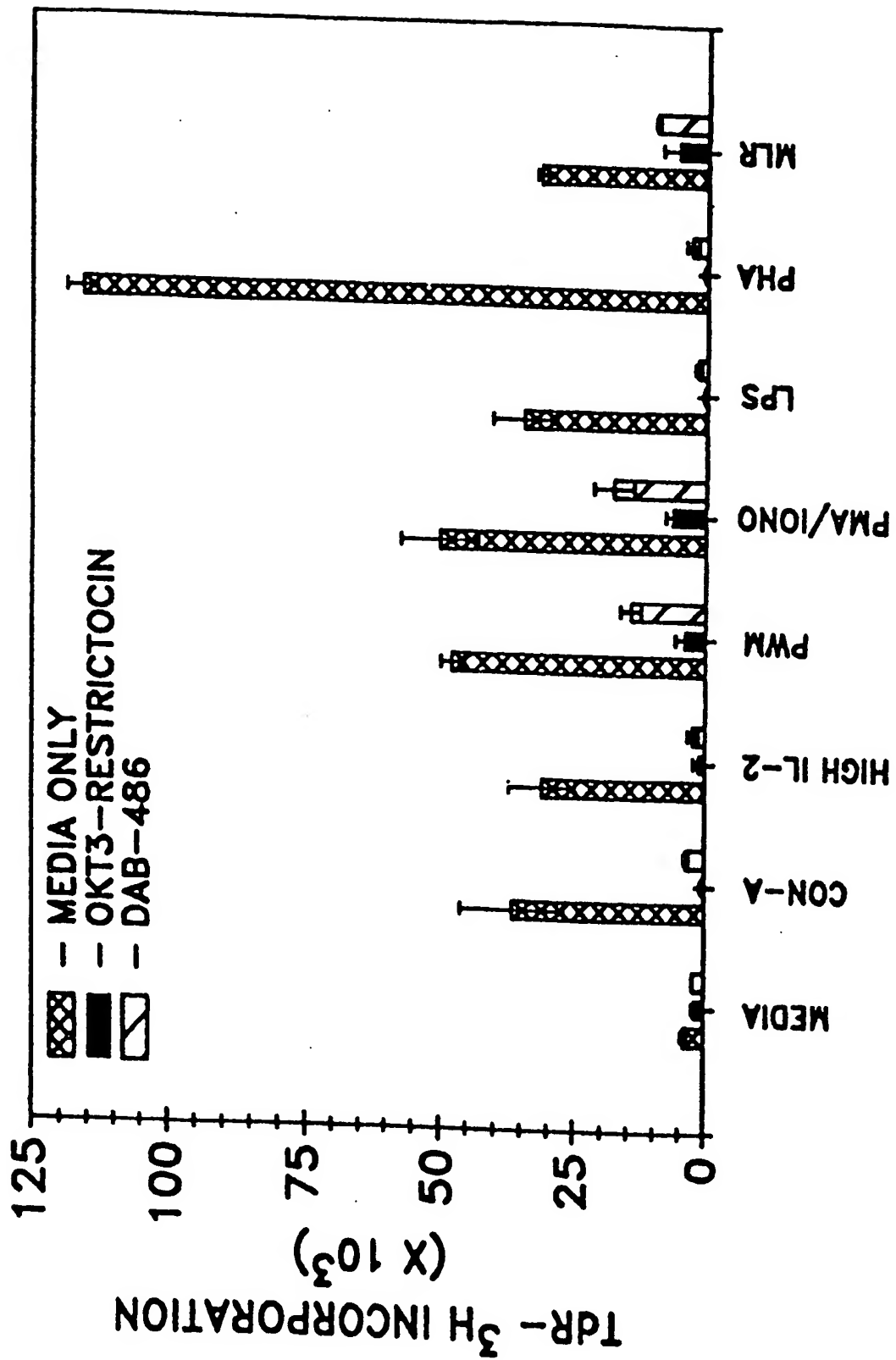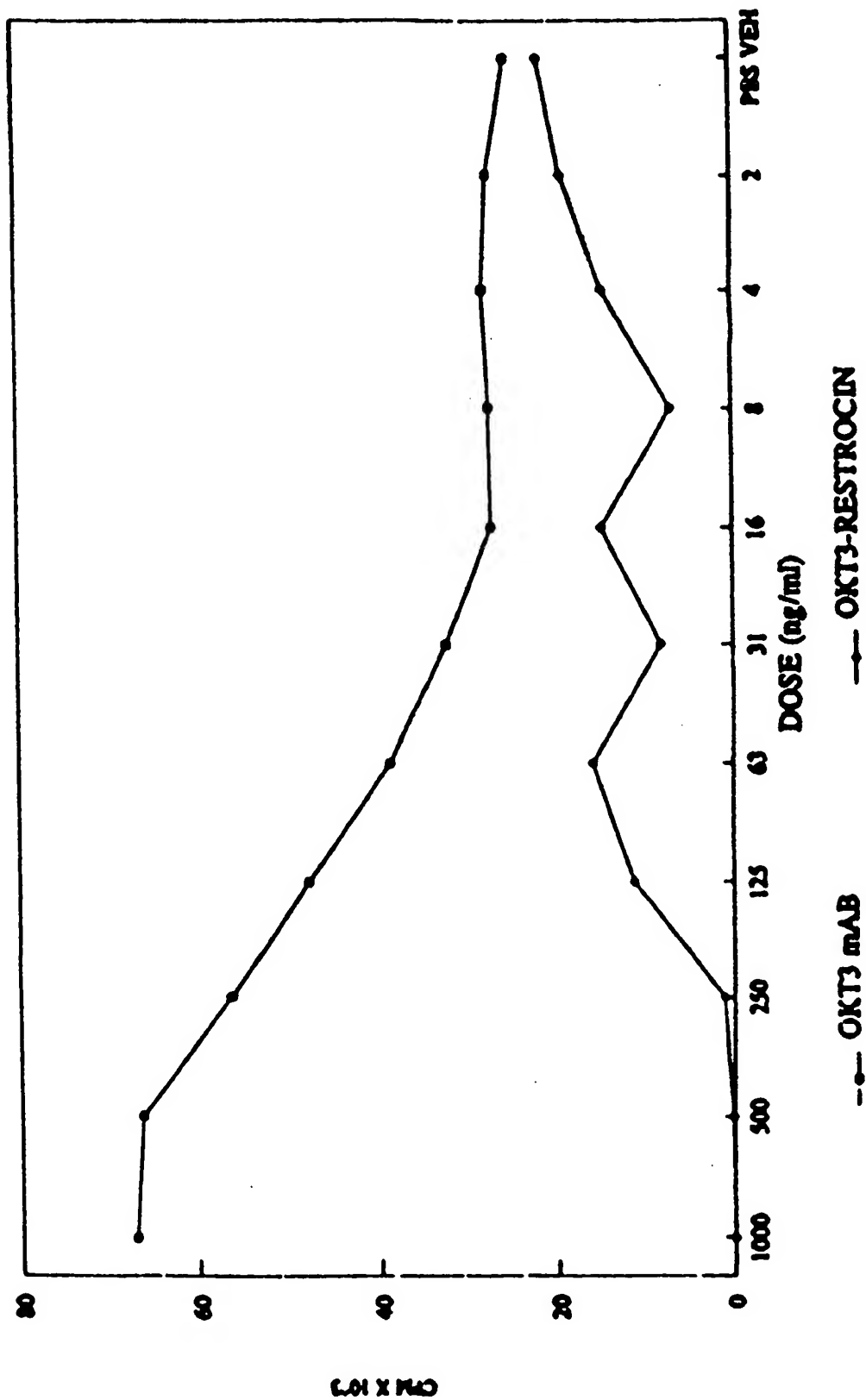an unprotected sulfhydryl group.

Figure 1

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/11963

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/28; A61K 39/395, 39/40, 39/42, 38/44
US CL : 530/391.7; 424/178.1, 179.1, 180.1, 181.1, 182.1, 183.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/391.7; 424/178.1, 179.1, 180.1, 181.1, 182.1, 183.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,629,194 A (DINSMORE) 13 May 1997, see entire document, especially column 15, lines 16-41 (note the recitation of OKT3 at line 30), column 8, line 53, and column 14, line 53 to column 15, line 65.	1-7
Y	RATHORE et al. Generation of Active Immunotoxins Containing Recombinant Restrictocin. Biochemical and Biophysical Research Communications. 06 May 1996, Vol. 222, No. 1, pages 58-63, see entire document, especially the abstract and page 59 first paragraph.	1-7
A	US 5,066490 A (NEVILLE Jr. et al) 19 November 1991, see entire document.	1-7

[X]	Further documents are listed in the continuation of Box C.	[ ]	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 JULY 1998	**0 3 SEP 1998**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JOSEPH W. RICIGLIANO
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,725,857 A (NEVILLE et al) 10 March 1998, see entire document.	1-7
A	BYERS et al. Use of an Anti-Pan T-Lymphocyte Ricin A Chain Immunotoxin in Steroid-Resistant Acute Graft-Versus-Host Disease. Blood. 01 April 1990, Vol. 75, No.7, pages 1426-1432, see entire document.	1-7

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11963

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★